IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:		
	Ronit EISENBERG, et al	
Serial No.:	10/009,809	
Filed:	April 26, 2002	
For:	Novel Anti-Allergic Agents	
Examiner:	Patrick J. Nolan	

Group Art Unit: 1644

Attorney Docket: **30836**

Commissioner for Patents
P. O. Box 1450
Alexandria VA 22313

DECLARATION OF RONIT SAGI-EISENBERG UNDER 37 CFR 1.132

I am presently employed as an Associate Professor at the Sackler Faculty of Medicine at Tel-Aviv University. I have more than 20 years of research experience in the fields of preclinical molecular biology research as well as clinical research in allergy and have published more than 40 papers in top scientific journals. Prior to my current appointment, I was a visiting scientist at the National Institute of Health (NIH), Bethesda MD and before that I held a position at the Weizmann Istitute of Science, Israel.

I am a co-inventor of the subject matter claimed in the above-referenced U.S. patent application.

I have read the Official actions issued with respect to the above-identified application.

In the current Official action, the Examiner has rejected claims 63 and 66-74 under 35 U.S.C §103 as being unpatentable over Kuby et al. in view of Aridor et al. and U.S. Pat. No. 5,807,746 to Lin. The Examiner states that Kuby et al. disclose that the inhibition of mast cell degranulation is a known mechanism to treat allergies. The Examiner further states that Aridor et al. teach the use of two peptides KNNLKECGLY and KENLKDCGLF in inhibiting mast cell degranulation when given to permeabilized cells, *in vitro* and that Lin teaches adding the sequence AAVALLPAVLLALLAP to any known biologically active peptide to allow transportation of the active peptide to the inside of the cell to allow for *in vivo* therapies. The Examiner concludes that one of ordinary skill in the art would be

motivated to add the importation peptides of Lin to the peptides taught by Aridor to treat allergies as treating allergies with mast cell degranulator inhibitors was well known in the art as taught by Kuby and that one of ordinary skill in the art would have a reasonable expectation of success in producing the claimed invention.

The Appendix section enclosed herewith shows using the teachings of the present invention an unprecedented efficacy of the claimed composition in treating a variety of mast cell associated disorders including asthma, skin allergy and ophthalmic allergy (both IgE dependent and IgE independent). Treatment of skin allergies with the peptide was as effective as the gold standard Cromoglycate and more potent than Fenistil Gel and Ceterizine. This peptide also effectively blocked IgE-independent conjunctivitis, showing similar efficacy as steroids and two-fold more efficacy than cromoglycate. Similar results were obtained in IgE-dependent conjunctivitis, wherein treatment was as effective as with commercially available drugs. In a rat model for asthma, treatment with the peptide significantly reduced bronchoconstriction.

These results conclusively show that the peptide of the present invention shows unexpected in vivo efficacy in various models of mast cell associated medical conditions.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United states Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

27 April 2006

Ronit Sagi-Eisenberg

Dr. Ronit Sagi-Eisenberg
Sackler Faculty of Medicine
Tel-Aviv University, Israel

Enc.:

CV of Dr. Ronit Sagi-Eisenberg; and
Appendix

April 2006

Curriculum Vitae
RONIT SAGI-EISENBERG, Ph.D.

Personal:
Born: December 24, 1952; Israel.
Nationality: Israeli
Marital Status: Married, +3.
Home address: 6 Lotus St. Ness-Ziona, 74045, Israel
Tel: 972-8-9405382

Education:

1970 - 1973:	B.Sc. in Chemistry, Cum Laude, Tel Aviv University, Israel.
1974 - 1975:	M.Sc. studies, Tel Aviv University, upgraded to Ph.D. studies.
1975 - 1980:	Ph.D. Studies. Ph.D. awarded May, 1981.

Positions Held:

1975 - 1980:	Assistant, Department of Biochemistry, Tel Aviv University, Israel.
10/80-10/84:	Postdoctoral fellow at the laboratory of Prof. I. Pecht, Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel.
08/82-11/82: 08/83-11/83:	Honorary Research Assistant at the Department of Pharmacology, University College London, U.K. Host: Dr. John C. Foreman.
10/84-10/85:	Investigator, Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel.
10/85-10/89:	Senior investigator, Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel.
10/89-8/91:	Associate Professor, Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel.
8/91-12/93:	Visiting scientist, Laboratory of Chemical Pharmacology, NHLBI, National Institutes of Health, Bethesda, MD, USA Host: Dr. Michael Beaven.
10/93-10/94:	Visiting professor, Department of Histology and Cell Biology, Sackler School of Medicine, Tel Aviv University, Israel.
10/94-present:	Associate professor, Department of Cell Biology and Histology, Sackler School of Medicine, Tel Aviv University, Israel.

Teaching:

1975 - 1980:	Teaching assistant at the Department of Biochemistry, Tel Aviv University.
1985, 1987, 1990:	Feinberg Graduate School of the Weizmann Institute of Science. Course on:"Transmembranal signalling pathways".
1994-present:	Sackler School of Medicine, Tel Aviv University. Course on Cell Biology.
1997, 1999, 2002:	Sackler School of Medicine, Tel Aviv University. Advanced Course on Signal Transduction and Protein Traffic.
2003, 2005:	Advanced Course: Molecular Mechanisms underlying Protein Trafficking.

HonorsandPersonalAwards:

1973: B.Sc. Cum Laude.
1974: Meier Foundation Award.
1976: Shenkar Foundation Award.
1977: Shenkar Foundation Award.
1978: Shenkar Foundation Award.
1979: Shenkar Foundation Award.
1979: The Michael Landau Prize for distinguished Ph.D. thesis.
1982: Michael Sela exchange fellowship for visiting the University of Toronto.
1982: British Royal Society - Israel Academy of Sciences program Award
1983: Mathilda Marks-Kennedy Scholarship.
1984: British Council Scholarship
1984: The "Bat Sheva de Rothschild" Fellowship Award for young distinguished investigators.
1985: Incumbent of Charles H. Revson Career Development Chair.
1985: International Cancer Research Technology Transfer (ICRETT) scholarship for visiting Dr. Wayne Anderson at the National Institutes of Health, USA.
1988: Israel Cancer Research Career Development Award.
1997: Sackler Faculty of Medicine Dean Award for Excellence in Teaching.
2002: Sackler Faculty of Medicine Dean Award for Excellence in Teaching.

AdministrativeDuties:

87-91: Member of the Teaching Committee of the Weizmann Institute of Science.
95-99: Member of the Institutional Animal Care and Use Committee.
96-99: Treasurer of the Israel Society for Cell Biology.
97-01: Preclinical Advisor for the Sackler School of Medicine, New York State/American Program.
98-01: Member of the Teaching Committee of the Sackler School of Medicine, New York State/American Program.
98-present: Member of the Research and Development Committee of the Sackler Faculty of Medicine.
98-present: Head of the Faculty of Medicine Committee for Laboratory Space.
98-present: Member of the Search Committee of the Sackler Faculty of Medicine.

2002-present: Member of the Teaching Committee of the school of Medicine
2002-present: Member of the University Committee of Intellectual property.
2002-present: Member of the Ph.D. students committee.
2005-present: Head of the Dept. of Cell and Developmental Biology.

MembershipinProfessionalAssociations:
The Israel Society for Cell Biology

FieldofInterest:
Signal Transduction, Protein Traffic, Allergic and Inflammatory diseases, Cancer

Students:
1986 - 1991: Dr. Galia Gat-Yablonski, Ph.D. student.
1987 -1991: Dr. Linton M. Traub, Ph.D. student.
1987 - 1993: Dr. Meir Aridor, M.Sc./Ph.D. student.
1995-1997: Mrs. Zipi Taube, MSc. Student.
1994-2000: Dr. Dana Baram, Ph.D. student (in collaboration with Prof. Y. Mekori).
1994-2000: Dr. Ariela Zussman, Ph.D. student.
1997-2000: Dr. Irit Shefler, Ph.D. student.
1998-2003: Dr. Elena Grimberg, Ph.D. student (in collaboration with Prof. I. Hammel).
2000-2005: Dr. Yafit Ettia-Nasagi, Ph.D. student.
2000-2005: Dr. Yara Kap-Barnea, M.Sc./Ph.D. student.
2000-2001: Dr. Ze Peng, Post Doctoral fellow.
2000-: Mr Semyon Melnikov, M.Sc./Ph.D. student.
2000-2005: Dr. Yael Haberman. M.D./Ph.D student.
2002-2004: Mrs. Hila Cohen, M,Sc. student.
2002-: Mrs. Carmit Kedar, Ph.D. student.
2002-: Mrs. Dana Lavi, M.Sc. student.
2003-: Ms. Idit Ziv, , Ph.D. student.
2005-: Ms. Anat benado, M.Sc. student.
2005-: Ms. Lihi Ninio, M.Sc. student.
2005-: Mrs. Dipannita Dutta, Ph.D. student.

InvitedLecturesatScientificMeetings:
1984: **15th Annual Meeting of the Israel Immunological Society, Tel-Aviv** , Israel, Lecture: "A regulatory role of protein kinase C in histamine release from basophils."
1986: **The Annual Conference of the Israeli Society for Endocrinology, Jerusalem** , Israel. Lecture: "The role of phosphoinositide metabolism in stimulus-secretion coupling."
1988: **UNESCO Meeting** on "Multiple Facets of Immunoregulation", Jerusalem, Israel. Lecture: "GTP-binding proteins as mediators of protein kinase C action."

1988: **EMBO-NATO-CEC Advanced Research Workshop on "The Guanine - Nucleotide Binding Proteins; Common Structural and Functional Properties", Renesse, The Netherlands.** Lecture: "A novel cytosolic GTP-binding protein with phospholipidstimulated GTP-binding and GTPase activity".

1989: **Annual Meeting of the Israeli Biochemical and Biophysical Societies , Rehovot, Israel.** Lecture: "Dual signalling by protein kinase C".

1990: **Symposium of the British Physiological Society on "The control of exocytosis", London, England.** Lecture: "Ligand activated but receptor independent exocytosis of mast cells; evidence for direct activation of G-proteins".

1990: **The 20th Annual Meeting of the Israeli Society for Immunology, Tel Aviv** , Israel. Lecture: "The role of GTP binding proteins in exocytosis".

1990: **Jacques-Monod Conference on Exocytosis, Aussois , France.** Lecture: "Receptor independent activation of mast cells by basic secretagogues; Evidence for direct activation of GTP-binding proteins".

1990: **Gentner Meeting on Cellular Signal Transduction Networks, Germany**. Lecture: "GTP binding proteins in protein trafficking".

1990: **The 20th FEBS Meeting, Budapest, Hungary.** Lecture: "GTP binding proteins as targets for protein kinase C actions".

1990: **The Meeting "From Receptor to Cellular Response", S. Maria Imbaro, Chieti, Italy,** Lecture: "Receptor-independent activation of mast cells by basic secretagogues; evidence for direct activation of Gproteins".

1990: **The International Conference on Leucocyte Biology in Crete, Greece.** Lecture: "The role of GTP-binding proteins in the regulation of exocytosis".

1991: Invited to the **4th Interscience World Conference on Inflammation, Geneva, Switzerland.**

1991: Invited to the **EMBO Techniques Course: "Techniques in Exocytosis", London, England.**

1992: **The Second Joseph Cohn Workshop on" Signal Transduction in Mast Cell Activation and Development", Ein-Gedi, Israel** . Lecture: " Receptor independent exocytosis in mast cells".

1993: **3rd G.I.F Meeting on Cancer and Immunopathology, Israel.** Lecture: "Signal Transduction and Vesicular Trafficking".

1993: **The annual meeting of the Israel Endocrine Society, Jerusalem,** Israel. Lecture: "The role of GTP-binding proteins in the control of membrane trafficking".

1995: **The meeting on "Signal transduction in the activation and development of mast cells and basophils", Washington, DC, USA**. Lecture: " Molecular mechanisms that control mast cell exocytosis; the role of the heterotrimeric G-protein Gi3".

1995: **The Israel Society for Cell biology; Minisymposium on intracellular trafficking, Tel-Aviv, Israel.** Lecture: "GTPbinding proteins in the control of exocytosis".

1995: **The meeting on:"New aspects on molecular neurosecretory mechanisms", Hamamatsu, Japan.** Lecture:"Molecular mechanisms that control mast cell exocytosis".

1995: **The "Advanced course on receptor pharmacology", Odense, Denmark.** Lecture: "Protein kinase C and diacylglycerol".

1996: **26th Annual Meeting of the Israel Immunological Society, Tel Aviv, Israel.** Lecture: ""Molecular mechanisms controlling secretion from mast cells".

1996: **ASBMB Fall Symposium on Integration of growth factor signalling pathways, Lake Tahoe, California.** Lecture: "Gi proteins activate protein tyrosine phosphorylation and release of arachidonic acid in mast cells via a novel protein kinase C and phosphatidylinositol 3-kinase-dependent mechanism".

1997: **The "Advanced course in receptor pharmacology", Aalborg, Denmark.**

1997: **Society of general Physiologists, Annual meeting on the: Mechanisms of Secretion, Woods Hole, Massachusetts.** Lecture: "Synaptotagmin as a calcium sensor in mast cell exocytosis"

1998: **Third International Workshop on Signal Transduction in the Activation and Development of Mast Cells and basophils. Washington, USA.** Lecture: The neuronal Ca2+ sensor synaptotagmin I regulates Ca2+-dependent exocytosis in mast cells.

2000: **The Fourth Symposium on Intracellular Trafficking, Tel Aviv. Israel.** Lecture: "Synaptotagmins and mast cell function".

2001: **Joint Meeting Tel Aviv University/Institute of Cancer Research, Vienna, Austria** . Lecture: "Synaptotagmin as a novel regulator of phorbol-ester-induced down-regulation of protein kinase C".

2001: **Membrane Dynamics in Endocytosis, Tomar, Portugal.** Lecture: "Synaptotagmins 2 and 3 as regulators of distinct endocytic pathways."

2001: **Fourth International Workshop on Signal Transduction in the Activationand Development of Mast Cells and Basophils, Bethesda, USA.** Lecture: Synaptic proteins in mast cell activation: A heretofore unrecognized participant in allergic responses.

2002: **Fourth International Symposium on Ocular Pharmacology and Pharmaceutics, Seville, Spain.** Lecture: New targets for allergy molecular level overview.

2002: **55th Harden Meeting on Dynamics of Membrane Traffic, Ambleside, UK.** Lecture: Synaptotagmins-Non-Redundant Regulators of Exo and Endocytic Pathways.

2002: **Study III Symposium, Tel Aviv.** Lecture: G-proteins as targets for the development of novel anti-allergic drugs

2002: **28th Meeting of FEBS, Istanbul, Turkey.** Lecture: G-proteins as targets for the development of novel anti-allergic drugs

2002: **1st International Workshop on Lysosome-Related Organelles , Portugal.** Lecture: Synaptotagmins-Non-Redundant Regulators of Exo and Endocytic Pathways.

2003: **Bio-Tech Israel 2003, Tel Aviv, Israel.** Lecture: G-proteins as targets for the development of novel anti-allergic drugs.

2003 **Joint Meeting of the Israel Societies for Cell Biology and Developmental Biology. Eilat, Israel.** Organizing Committee.

2003 **Membrane Dynamics in Endocytosis, Acquafredda di Maratea (near Naples), Italy.** Lecture: Synaptotagmins as traffic regulators to and from the endocytic recycling compartment (ERC)."

2003 **Israel Society for Physiology and Pharmacology,** Maale Hahamisha, Israel. Lecture: Synaptotagmins as traffic regulators to and from the endocytic recycling compartment (ERC)."

2004: **2nd International workshop on lysosome-related organelles , Portugal.** Invited lecture.

2005: The Batsheva de Rothschild International Workshop on Mast cell signalling and function in health and disease, Eilat, Israel. Lecture: 'Neuronal calcium sensor-1 (NCS-1), a novel regulator of FcεRI-induced signaling in mast cells".

2005: **International Symposium on Signal Transduction in Health and Disease Tel Aviv, Israel.** Lecture: "Neuronal calcium sensor-1 (NCS-1), a novel regulator of FcεRI-induced signaling in mast cells".

2005: **Reseach Fair, Tel Aviv University.** Lecture: "Molecular mechanisms underlying mast cell triggering".

2005: **Membrane Dynamics in Endocytosis, Sant Feliu de Guixols, Spain.** Poster: "Neuronal Calcium Sensor-1 (NCS-1) Stimulates and Extends ERK1/2 Signaling by Accelerating ERK1/2 Recycling through the Endocytic Recycling Compartment (ERC)".

2006: **Cell Signaling World Meeting, Luxemburg.** Poster: "Exploring the chemotherapeutic benefit of combining Gemcitabine (Gemzar) and Rapamycin".

2006: **Joint meeting of the Israeli Societies for Cell and Developmental Biology. Eilat, Israel.** Lecture: "Synaptotagmins, the gate keepers of endocytic networks".

Grants awarded (past 5 years):

1) The Tyssen Foundation: "Mechanism of neurogenic inflammation" 03/1997-03/2000 $35,000/year

2) The Israel Science Foundation: "The possible role of the synaptic vesicle Ca2+ sensor, synaptotagmin, in regulating mast cell exocytosis" 10/1997-10/2000 $44,688/year

3) The Israel Science Foundation: Equipment grant-Imaging system for gels and blots. 07/1998.

4) Tel Aviv University Internal Fund: " Elucidating the mechanisms of trafficking of the Synaptotagmin (Syt) homologs, Syt I and Syt II, to secretory granules and lysosomes, respectively". 1/08/1999-31/07/2000 $ 12,500.

5) The Israel Ministry of Health: "The role of Synaptotagmin II in controlling lysosome exocytosis in mast cells". 09/1997-10/2001 IS 60,000.

6) The Ministry of Trades and Industry: Foundation of "**Allergene Ltd.**" A company within the framework of Rad-Ramot-High Technologies Incubator. Project goal: The development of an antiallergic compound for skin allergies.

06/1998-08/2000 $180,000/year
7) The Recanati Foundation: Synaptic Proteins in Mast Cell Activation: a Heretofore Unrecognized Participant in the Allergic Response.
4/2000-9/2001 $25,000
8) The Israel Science Foundation: "The role of synaptotagmin III in regulating mast cell exocytosis"
10/2000-10/2004 $50,000/year
9) Tel Aviv University Cancer Center: "Synaptotagmin II and the trimeric GTP-binding protein Gi3 as novel regulators of Cathepsin D trafficking in rat basophilic leukemia cells".
09/00-09/01 $15,000.
10) The Israel Science Foundation: The role and mode of action of Synaptotagmin (Syt) IX in controlling exit from the endocytic recycling compartment.
10/04-10/08 $75,000/year
11) Ramot/Biolight: Development of anti allergic drugs.
01/06-12/06-$100,000
12) Bio-Disc –Cooperation with Germany: "Regulators of endocytosis and trafficking".
02/06-02/08 Euro 96,000

Listofpublications:

1. Sagi-Eisenberg, R. and Gutman, M. "Generation of high $\Delta\Psi$ in Respiring Submitochondrial Particles by Steady-State Accumulation of Oxidized N,N,N',N' - Tetramethyl-p-phenylenediamine". *Eur. J. Biochem.* **97**, 127-132 (1979).

2. Sagi-Eisenberg, R. and Gutman, M. "Rate Limiting Step in Oxidation of Physiological and Artificial Reductants by Azotobacter Vinelandii Membrane Vesicles". Arch. Biochem. Biophys. **197**, 470-476 (1979).

3. Sagi-Eisenberg, R., Ben-Neriah, Z., Pecht I., Terry S. and Blumberg S. "Structure Activity Relationship in the Mast Cell Degranulating Capacity of Neurotensin Fragments".*Neuropharmacology* **22**, 197-201 (1983).

4. Sagi-Eisenberg, R. and Pecht, I. "Membrane Potential Changes During IgE-Mediated Histamine Release from Rat Basophilic Leukemia Cells (RBL)". *J. Memb. Biol.* **75**, 97-104 (1983).

5. Sagi-Eisenberg R., Geller-Bernstein C., Ben-Neriah Z. and Pecht I. "Direct Measurement of the Dextran-Dependent Calcium Uptake by Rat Peritoneal Mast Cells". *FEBS Lett.* **161**, 37-40 (1983).

6. Sagi-Eisenberg, R. and Pecht, I. "Resolution of Cellular Compartments Involved in Membrane Potential Changes Accompanying IgE-Mediated Degranulation of Rat Basophilic Leukemia Cells". *EMBO J.* **3**, 497-500 (1984).

7. Sagi-Eisenberg, R. and Foreman, J.C. "Fractionation of Mast Cell Components for studies of Ligand-Receptor Binding at the Plasma Membrane". *Immunol. Lett.* **8**, 43-47 (1984).

8. Sagi-Eisenberg, R. and Pecht, I. "Protein Kinase C, a Coupling Element between Stimulus and Secretion in Basophils". *Immunol. Lett.* **8**, 237-241 (1984).

9. Sagi-Eisenberg, R., Mazurek, N. and Pecht, I. "Ca2+ Fluxes and Protein Phosphorylation in Stimulus-Secretion Coupling of Basophils". *Molec. Immunol.* **21**, 1175-1181 (1984).

10. Sagi-Eisenberg, R. "A Possible Role for a Calcium Activated, Phospholipid Dependent Protein Kinase in the Mode of Action of the Anti-Allergic Drug Disodium Cromoglycate". *Trends Pharmacol. Sci.* **6**, 198-201 (1985).

11. Sagi-Eisenberg, R., Lieman H. and Pecht I. "Protein Kinase C Regulation of the Receptor Coupled Calcium Signal in Histamine Secreting Rat Basophilic Leukemia Cells". *Nature* **313**, 59-60 (1985).

12. Sagi-Eisenberg, R., Foreman, J.C. and Shelly, R. "Histamine release induced by histone and phorbol ester from rat peritoneal mast cells". *Eur. J. Pharmacol.* **113**, 11-17 (1985).

13. Tarrab-Hazdai, R., Sagi-Eisenberg, R., Brenner, V. and Arnon, R. "Ion fluxes changes during early stages of Schistosoma mansoni; Evaluation of complement effect". *Eur. J. Biochem.* **154**, 563-568 (1986).

14. Zick, Y., Sagi-Eisenberg, R., Pines, M., Gierschik, P. and Spiegel, A.M. "Multi-site phosphorylation of the alpha subunit of transducin by the insulin receptor kinase and protein kinase C". *Proc. Natl. Acad.Sci. USA*, **83**, 9294-9297 (1986).

15. Reck, B., Sagi-Eisenberg, R. and Pecht, I. "Cytosolic free Ca2+ in mast cells and their mediators release". *J. Allergy Clin. Immunol.*,164-169 (1986).

16. Sagi-Eisenberg, R., Foreman, J.C., Raval, P.J. and Cockcroft, S. "Protein and diacylglycerol phosphorylation in the stimulus secretion coupling of rat mast cells." *Immunology*, **61**, 203-206 (1987).

17. Zick, Y., Spiegel, A.M. and Sagi-Eisenberg, R. "Insulin-like growth factor I receptors in retinal rod outer segments". *J. Biol. Chem.* **262**, 10259-10264 (1987).

18. Safran, A., Sagi-Eisenberg, R., Neuman D. and Fuchs S. "Phosphorylation of the acetylcholine receptor by protein kinase C and identification of the phosphorylation site within the receptor d-subunit". *J. Biol. Chem.* **262**, 10506-10512 (1987).

19. Sagi-Eisenberg, R. "GTP-binding proteins as possible targets for protein kinase C action. *Trends Biochem. Sci.* **14**, 355-357 (1989).

20. Sagi-Eisenberg, R., Traub, L.M., Spiegel, A.M. and Zick, Y. "Protein kinase C mediated phosphorylation of retinal rod outer segment membrane proteins". *Cell. Signalling* **1**, 519-531 (1989).

21. Safran, A., Provenzano, C., Sagi-Eisenberg, R. and Fuchs, S. "Phosphorylation of membrane-bound acetylcholine receptor by cAMP-dependent protein kinase and protein kinase C; Characterization and subunit specificity". *Biochemistry* **29**, 6730-6734 (1990).

22. Gat-Yablonski, G. and Sagi-Eisenberg, R. "Evaluation of the role of inositol trisphosphate in IgE-dependent exocytosis". *Biochem. J.* **270**, 685-689 (1990).

23. Gat-Yablosnki, G. and Sagi-Eisenberg, R. "Differential down-regulation of protein kinase C selectively affects IgE-dependent exocytosis and inositol trisphosphate formation". *Biochem. J.* **270**, 679-684 (1990).

24. Aridor, M., Traub, L. and Sagi-Eisenberg R. "Exocytosis in mast cells by basic secretagogues; Evidence for direct activation of GTP-binding proteins". *J. Cell Biol.* **111**, 909-917 (1990).

25. Traub, L.M., Evans, H.W. and Sagi-Eisenberg, R. "A novel 100 kDa protein, localized to receptor enriched endosomes, is immunologically related to the signal transducing G proteins Gt and Gi." *BiochemJ.*, **272**, 453-458 (1990).

26. Zick, Y. and Sagi-Eisenberg, R. "A combination of H2O2 and vanadate concomitantly stimulates protein tyrosine phosphorylation and polyphosphoinositide breakdown in different cell lines". *Biochemistry* **29**, 10240-10245 (1990).

27. Aridor, M. and Sagi-Eisenberg, R. "Neomycin is a potent secretagogue of mast cells that directly activates a GTP-binding protein involved in exocytosis". *J. Cell Biol.* **111**, 2885-2891 (1990).

28. Traub, L.M., Shai, E. and Sagi-Eisenberg, R. "Characterization of the interaction between p100, a novel G protein-related protein, and rat liver endosomes". *Biochem J.* **280**, 171-178 (1991).

29. Traub, L.M., and Sagi-Eisenberg, R. "Purification of p100, a protein antigenically related to the signal transducing G proteins Gt and Gi; Evidence for an adaptin like protein". *J. Biol. Chem.* **266**, 24642-24649 (1991).

30. Hulkower, K.I., Sagi-Eisenberg, R., Traub, L.M., Georgescu, H.I. and Evans, C.H. " Interleukin-1 and synovial protein kinase C: Identification of a novel, 35kDa cytosolic substrate". Agents and Actions **34**, 278-281 (1991).

31. Hulkower, K.I., Sagi-Eisenberg, R., Traub, L.M., Georgescu, H.I. and Evans, C.H. " Synovial protein kinase C and its apparent insensitivity to interleukin-1". *Eur.J. Biochem.* **209**, 81-88 (1992).

32. Aridor, M., Rajmilevich, G., Beaven, M. and Sagi-Eisenberg, R. "Activation of exocytosis by the heterotrimeric G-protein Gi3 "*Science.* **262**, 1569-1572 (1993).

33. Hydar, A., Maeyama, K., Sagi-Eisenberg, R. and Beaven, M.A. "Antigen and Thapsigargin promote influx of Ca2+ in rat basophilic RBL-2H3 cells by ostensibly similar mechanisms that allow filling of inositol 1,4,5-trisphosphate-senesitive and mitochondrial Ca2+ stores". *Biochem. J.* **304**, 431-440 (1994).

34. Kassessinoff, T.A., Gabet, A., Beaven, M.A. and Sagi-Eisenberg, R. "Inositol polyphosphates regulate the membrane interactions of the endosomal p100, G-Protein-related protein". *Biochim. Biophys. Acta.* **1394**, 111-120 (1998).

35. Baram, D., Linial, M., Mekori, Y.A. and Sagi-Eisenberg, R. "Ca2+- dependent exocytosis in mast cells is regulated by the Ca2+ sensor Synaptotagmin I". *J. Immunol. (Cutting Edge)* **161**, 5120-5123 (1998).

36. Shefler, I., Taube, Z., Medalia, O. and Sagi-Eisenberg, R. "Basic secretagogues activate protein tyrosine phosphorylation and release of arachidonic acid in mast cells via a novel protein kinase C and phosphatidylinositol 3-kinase-dependent mechanism" *Eur. J. Immunol.* **28**, 3468-3478 (1998).

37. Zussman, A., Hermuet, S. and Sagi-Eisenberg, R. "Stimulation of Ca2+-dependent exocytosis and arachidonic acid release in cultured mast cells (RBL-2H3) by a GTPase-deficient mutant of Gαi3." *Eur. J. Biochem.* **258**, 144-149 (1998).

38. Shefler, I., Seger, R. and Sagi-Eisenberg, R. "Gi-mediated activation of the mitogen-activated protein kinase (MAPK) pathway by the receptor mimetic basic secretagogues of connective tissue type mast cells. Bifurcation of arachidonic acid-induced release upstream of MAPK." *J. Pharmacol. Exp. Ther.* **289**, 1654-1661 (1999).

39. Baram, D., Adachi, R., Medalia, O., Tuvim, M., Dickey, B.F., Mekori, Y.A..and Sagi-Eisenberg, R. "Synaptotagmin II negatively regulates Ca2+-triggered exocytosis of lysosomes in mast cells". *J. Exp. Med.* **189**, 1649-1658 (1999).

40. Zussman, A. and Sagi-Eisenberg, R. "Stimulation of Ca2+-Dependent Exocytosis and Release of Arachidonic Acid in Cultured Mast Cells (RBL-2H3) by Quercetin; Sensitization, Linked to Inhibition of Gi3 GTPase Activity". *International Journal of Immunopharmacology* **22**,
747-754 (2000).

41. Shefler, I. and Sagi-Eisenberg, R "Gi-mediated of the Syk Kinase by the Receptor Mimetic Basic Secretagogues of Mast Cells; Role in Mediating Arachidonic Acid/Metabolites Release. J. Immunol. **167**, 475-481 (2001).

42. Shefler, I. and Sagi-Eisenberg, R "Gi-mediated of the Activation of the p42/p44 Mitogen-Activated Protein Kinases by Receptor Mimetic Basic Secretagogues is Abrogated by Inhibitors of Endocytosis. International Immunopharmacology. **2**, 711-720 (2002).

43. Baram, D., Peng, Z., Medalia, O., Mekori, Y.A. and Sagi-Eisenberg, R. "Synaptotagmin II Negatively Regulates MHC class II Presentation by Mast Cells". Molecular Immunol. **38**, 1347-1352 (2002).

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APPENDIX 1

Commonly used cell penetrating peptides

(Yellow marks proline at c')

1.	AntpPTD Drosophila	RQIKIWFQNRRMKWKK
2.	FtzPTD Drosophila	RQIKIWFQNRRMKSKK
3.	Engrailed PTD Drosophila	RQIKIWFQNKRAKIKK
4.	Hox-A5 PTD human	RQIKIWFQNRRMKWKK
5.	Isl-1 PTD human	RVIRVWFQNKRCKDKK
6.	Transportan	AGYLLGKINLKALAALAKKIL
7.	Transportan (2)	GWTLNSAGYLLGKINLKALAALAKISIL
8.	MTS peptide kFGF based	KGEGAAVLLPVLLAAPG
9.	Tat	CGRKKRRQRRRPPQC
10.	pVEC	LLIILRRRIRKQAHAHSK
11.	MAP	KLALKLALKALKAALKLA
12.	MPG	GALFLGFLGAAGSTMGAWSQPKSKRKV
13.	GALA	WEAALAEALAEALAEHLAEALAEAEALEALAA
14.	KALA	WEAKLAKALAKALAKHLAKALAKALAKACEA
15.	JTS-1	GLFEALLELLESLWELLLEA
16.	HA	GLFEAIAGFIENGWEGMIDG
17.	Lear	GLFEAIAGFIENGWEGMIDGWYG
18.	K5	GLFKAIAKFIKGGWKGLIKG
19.	E5	GLFEAIAEFIEGGWEGLIEG
20.	H5WYG	GLFHAIAAHFIHGGWHGLIHGWYG
21.	E5CA	GLFEAIAEFIEGGWEGLIEGCA
22.	E5WYG	GLFEAIAEFIEGGWEGLIEGWYG
23.	INF-1	GLFEAIAGFIENGWEGMIDGGGC
24.	INF-7	GLFEAIEGFIENGWEGMIDGWYG
25.	adenoviral peptide m	MRRAHHRRRRASHRRMRGG
26.	IP/K-FGF	AAVALLPAVLLALLAP
27.	Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ
28.	Tat gp41	GRKKRRQRRRPPQC
29.	Ant Homeodomain	RQIKIWFQNRRMKWKKC
30.	SV-40 gp32	PKKKRKV
31.	Influenza nucleoprotein	NSAAFEDLRVLS
32.	NF-kB p50	VQRKRQKLM
33.	Grb2 SH2 domain	AAVLLPVLLAAP
34.	Integrin b3	VTVLALGALAGVGVG
35.	Short minimal TAT	YGRKKRRQRRR
36.	Poly-arginine Basic	(R)7-11
37.	VP22	DAATATRGRSAASRPTQRPRAPARSASRPRRPVQ
38.	PEP-1	KETWWETWWTEWSQPKKKRKV
39.	P1	MGLGLHLLVLAAALQGAWSQPKKKRKV
40.	hCT(9-32)	LGTYTQDFNKFHTFPQTAIGVGAP
41.	JTS1	GLFEALLELLESLWELLLEA

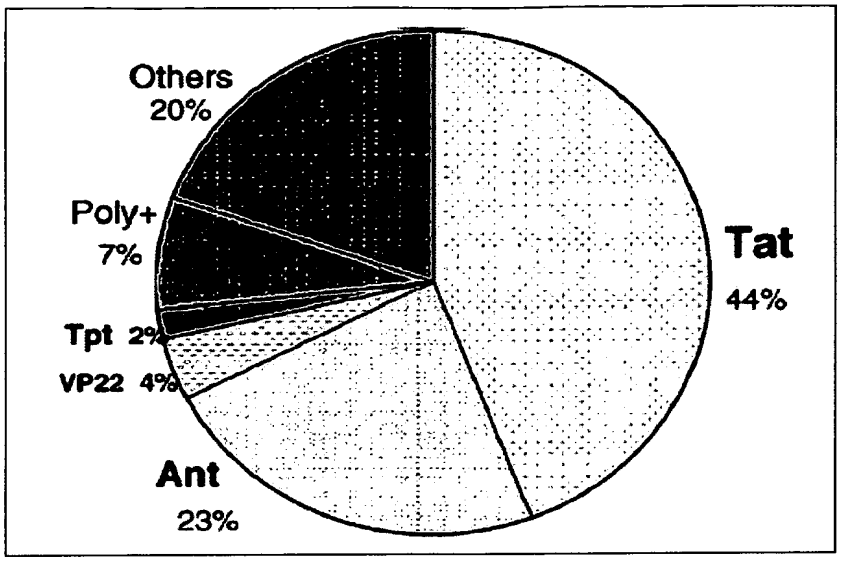

Fig. 1. Representation of the occurrence for the study and the use of various cell penetrating peptides (CPP) described in the review of Dietz and Bahr and entitled Delivery of bioactive molecules into the cell: the Trojan horse approach. Tat stands for the Tat derived peptide, Ant for the Antennapedia derived peptide or Penetratin, VP22 for the VP22 protein from herpes virus, Tpt for Transportan and poly+ for cationic peptides such as poly-lysine or poly-arginine.

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Noninvasive intracellular delivery of functional peptides and proteins

Jacek Hawiger

In order to probe intracellular signaling based on interactions of thousands of proteins expressed in the living cell, new methods of noninvasive delivery of functional peptides and proteins to cells have been developed. These include cellular import of peptides and proteins based on the cell-membrane-permeable properties of the hydrophobic region of a signal peptide sequence. The prototypical cell-permeable SN50 peptide, which contains the nuclear localization signal sequence of NK-κB p50, has been applied in multiple cell types to block nuclear import of this and other transcription factors. Further developments, including site-specific ligation of bipartite import peptides and production of import-competent recombinant proteins, provide the means for easy and rapid delivery of peptides and proteins to a wide spectrum of cells in order to regulate intracellular pathways involved in adhesion, signaling and trafficking to the nucleus.

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Abbreviations

CARD	cell adhesion regulatory domain
ECV	human endothelial
EGF	epidermal growth factor
HEL	human erythroleukemia
h region	hydrophobic core region
MPS	membrane-permeable sequence
NLS	nuclear localization signal
SH2	src homology 2
SN50	NLS of NF-κB p50

Introduction

The emerging field of proteomics is based on the startling realization that the average nucleated cell contains approximately 10,000 proteins that participate in signal transduction, gene transcription, cell–cell communication and intracellular protein trafficking. These functions of the living cell depend on intracellular protein–protein, protein–lipid and protein–DNA interactions involving more or less defined domains or consensus sequences. The ongoing human genome sequencing effort continually yields new structural information about these proteins. Their structure/function analysis, however, is hampered by the inability of analytical tools such as sequence-specific antibody probes or synthetic peptides to traverse the plasma membrane barrier. Therefore, invasive techniques of microinjection or applications of membrane-disrupting pore-forming reagents [1,2] are usually employed to introduce antibodies, synthetic peptides or other noncell membrane-permeable molecules into cells. Alternatively, transfection experiments are employed to introduce DNA encoding truncated or mutated intracellular proteins [3]. While such approaches yield significant information, their inherent limitations impede structure/function analysis of existing and newly sequenced intracellular proteins.

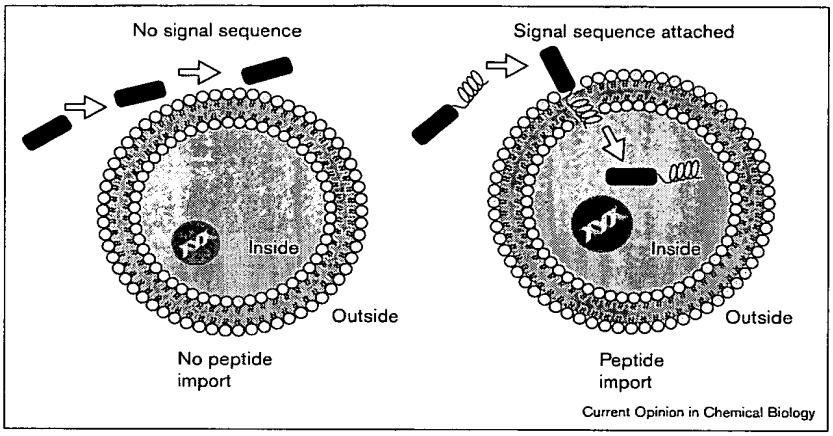
To overcome these limitations and allow easy, noninvasive delivery of peptides and proteins to intact cells in bulk concentration, new methods have been developed (see [1] for a review). One such technique utilizes the core hydrophobic region (h region) of a signal peptide sequence as a 'carrier' for cellular import of relevant segments or motifs of intracellular proteins [4,5]. Initially, the hydrophobic membrane-permeable peptide was grafted onto a functional segment representing an intracellular protein or its mutant by conventional step-wise solid-phase peptide synthesis, thus creating cell-permeable peptides with hydrophobic and functional domains linked by a normal peptide bond [4,5]. Most recently, two developments have expanded potential applications of noninvasive delivery of peptides and proteins to living cells. A facile method that expedites synthesis of bioactive cell-permeable peptides by site-specific ligation of two free peptides modules has been developed [6••]. This method is based on formation of a nonpeptide thiazolidine linkage between the membrane-permeable sequence (MPS) module and the functional sequence module. The second development expands the use of the signal sequence h-region-based delivery method to recombinant fusion proteins [7••]. In this work, glutathione *S*-transferase (GST) was fused to the Grb2 src homology 2 (SH2) domain and delivered to NIH3T3 fibroblasts using an expressed motif based on a modified MPS [7••].

In this brief review, I will discuss primarily a noninvasive method, based on the h region of signal peptides, to deliver functional peptides and proteins into cells. The application of this approach encompasses intracellular probing of signaling to the nucleus and structure/function analysis of cytoplasmic segments of cellular receptors.

Crossing of the cell membrane barrier by peptides and proteins

The plasma membrane of eukaryotic cells is inherently impermeable to peptides and proteins that lack specialized membrane receptors or transport proteins. These mechanisms of internalization are based on receptor-mediated endocytosis or transporter-based uptake. The endocytic mechanism is involved in internalization of cationized polylysine-based macromolecules such as loligomers [8]. Specialized transporters such as the H^+-dependent high-affinity transport system (PEPT) are utilized as channels

Figure 1



The conceptual design of peptide import based on signal sequence. Cell-permeable peptides are designed by using the h region of the signal peptide (represented by the helix-like leading portion) covalently bound to the amino terminus or the carboxyl terminus of selected sequences of intracellular proteins.

for the uptake of very short peptides [9]. Nevertheless, some viral and bacterial proteins are endowed with properties to cross the plasma membrane and gain entry into the living cell. These include the Herpes virus 22 protein VP22 and the tat protein of HIV, as well as a number of bacterial toxins that kill eukaryotic cells by punching holes in their membranes [10•,11–13]. Such toxins form channels in the plasma membrane, allowing toxic principle to enter the cell. Examples of such an entry by bacterial toxins include a thiol-activated clostridial toxin, perfringolysin O and anthrax toxin-protective antigen [13,14].

The principle of noninvasive delivery method based on the hydrophobic core region of signal peptides

In collaboration with Lin *et al.* [4], we have developed a new noninvasive method of delivery of functional peptides into a cell using a signal peptide as an importing vehicle. The rationale underlying this method was that the hydrophobic sequence of the signal peptide known to translocate through cell membranes and phospholipid vesicles could serve as a 'leading edge' to carry peptides and proteins into cells. The h region, comprising 7–16 nonconserved amino acid residues, is the dominant structure determining membrane-translocating signal sequence function. Importantly, the h region has the most persistent helix conformation with respect to other domains in the signal peptide, often seen, for, example, as a helix-turn-helix motif; the correlation of the stability of this conformation with *in vivo* function is clearly seen with the prokaryotic LamB protein [15]. The h region was identified in 126 signal peptides ranging in length from 18–21 residues [16]. The ability of the signal peptides to insert into membranes and their *in vivo* function correlate with the residue-average hydrophobicity of their hydrophobic cores. This is the critical characteristic of signal sequences, even though they lack primary sequence identity [17]. It has been shown that neither the positively charged N region (the amino-terminal segment) of the signal sequence nor the C region (the signal peptidase cleavage site) is necessary for membrane translocation of preprolactin [18].

The conceptual depiction of signal-peptide-based 'outside–in' delivery of functional molecules is illustrated in Figure 1. Functional peptides without a membrane-translocating 'leading edge' remain outside the cells because they cannot cross the peptide-impermeable membrane barrier. Synthetic peptides carrying signal sequence will cross cell membrane. This hypothesis was successfully tested in our laboratories using two distinct signal sequences derived from Kaposi fibroblast growth factor and from integrin β_3 [4,5]. The amino-terminal positively charged N region and the carboxy-terminal cleavage site were deleted from both signal peptide sequences. The remaining h region thereby constitutes the MPS. The carboxy-terminal end of MPS is linked through an amide or nonamide bond to a functional cargo [4,6••]. An epitope tag is included in order to detect the imported peptides in intracellular compartments using monospecific antipeptide antibodies.

Cellular import of signal sequence h-region-engineered peptides is concentration- and temperature-dependent [4] and independent of cell type [1]. The list of cells competent for import of signal-sequence-engineered peptides tested by us and others includes five human cell types: monocytic, endothelial, T lymphocyte, fibroblast and erythroleukemia, and three murine lines (Table 1). The imported peptides carrying functional cargo change the intracellular signaling pathways in these cells in a significant way. Analysis of two different signaling pathways illustrate the power of cell-permeable peptide import applied by us to identify a new functional domain in the intracellular segment of integrins and to probe nuclear import of four transcription factors (described below) [4,5,19••].

Table 1

Noninvasive delivery of peptides and proteins based on the h region of the signal sequence.

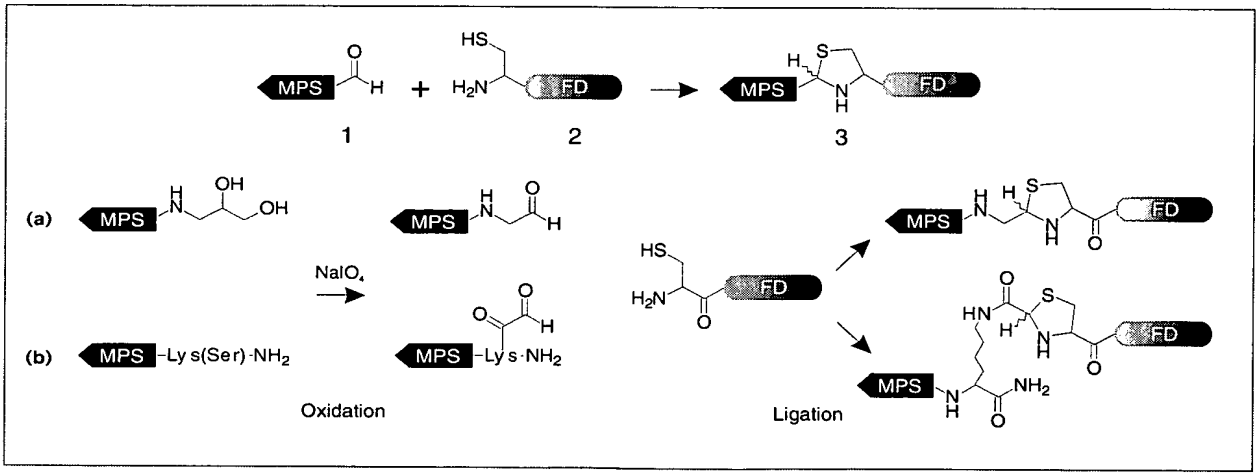
MPS	Functional domain	Cell line	Reference
AAVALLPAVLLALLAP*	SN50 peptide	Human monocytic THP-1 cells	[4]
		Human endothelial cells	[28]
		Murine endothelial LE-II cells	[4]
		Human T lymphocytes Jurkat	[6••,19••]
		Human fibroblasts	[27]
	Nuclear-localized sequence of USF2	Murine mast cells	[29]
AAVLLPVLLAAP	Grb2 SH2 domain	Murine NIH3T3 cells	[7]
VTVLALGALAGVGVG	Integrin β_3 cytoplasmic domain	Human erythroleukemia HEL cells	[5,6••]
	Integrin α_{IIb} cytoplasmic domain	Human endothelial ECV 304 cells	[5]
	Integrin β_1 cytoplasmic domain	Human fibroblasts cell line	[5]

*Single letter amino acid code. USF2, upstream stimulating factor 2.

Most recently, in collaboration with Tam and co-workers [6••], we have developed a facile method that expedites synthesis of biologically active, cell-permeable peptides by site-specific ligation of two free peptide modules, one bearing a functional sequence and the second bearing an MPS. A nonpeptide thiazolidino linkage between the two modules is produced by ligation of the carboxy-terminal aldehyde on the MPS and the amino-terminal 1,2-amino thiol moiety on the functional domain (see Figure 2). Stoichiometric amounts of fully unprotected MPS and functional peptide in an aqueous buffered solution are used with a high yield, thereby eliminating the need for additional chemical manipulation and purification prior to use in bioassays [6••]. Two different MPSs were interchangeably combined with two different functional sequences to generate two sets of hybrid peptides. In each assay, these peptidomimetic hybrids were found to be functionally comparable to peptides prepared by the conventional method. Cumulatively, this new ligation approach provides an easy and rapid method to engineer functional, cell-permeable peptides and demonstrates the potential for synthesis of cell-permeable peptide libraries designed to block intracellular protein–protein interactions.

The intracellular localization of imported peptides was verified by a number of criteria (e.g. inaccessibility to extracellular proteases, confocal laser scanning microscopy using monospecific antipeptide antibodies and functionality of imported

Figure 2



A general scheme for peptide ligation via thiazolidine ring formation using unprotected peptides. Ligation of **1**, a membrane-permeable peptide aldehyde, with **2**, an amino-terminal cysteinyl functional domain (FD), yields **3**, the ligation product linked by a thiazolidine ring. **(a)** Thiazolidine ring formation between the carboxy-terminal peptide glycoaldehyde and the amino-terminal cysteinyl peptide. **(b)** Thiazolidine ring formation between the lysinyl sidechain aldehyde group and the amino-terminal cysteinyl peptide. Reproduced with permission from, and for futher details see, [6••].

peptides designed to inhibit intracellular protein–protein interactions) [4,5,6••,19••]. The functional effects of imported peptides were sequence-specific (i.e. imported peptides carrying wild type sequence motifs of intracellular protein are functional, whereas those carrying mutated sequences were nonfunctional) [4,5••,19••]. Peptide import rapidly reached a maximum within 45 min at 37°C. The intracellular concentration of imported peptide reached 4% of the peptide added to the media, as determined by counting cell-associated ^{125}I-labeled peptides or by antipeptide antibody in cell enzyme-linked immunosorbent assays [4,19••]. The imported peptide was detectable in cells for up to 180 min [4,5,19••].

The mechanism of membrane translocation of signal-sequence-containing polypeptides

It is not presently known whether the characteristics of membrane translocation involved in the export of proteins whose signal sequence is cleaved by a signal peptidase in the endoplasmic reticulum lumen are applicable to the mechanism of peptide importation to the cell mediated by the h region of a signal peptide. We have established that, while cell-permeable peptide import is mediated by the h region, it is h-region-nonspecific, as synthetic peptides containing the h region from distinct protein signal sequences (e.g. kaposi fibroblast growth factor and the human integrin β_3 subunit) can translocate the plasma membrane [4,5]. Second, cell-permeable peptide import is not limited to a particular cell type, as it is readily accomplished in several human and animal cell lines (described earlier). This characteristic distinguishes it, for example, from cell-type-dependent import of the antennapedia homeobox peptide [20]. Although the internalization method of this peptide is receptor-independent it seems to require expression of α-2,8-polysialic acid, which decorates neuronal cell adhesion molecules (NCAMs) [21,20]. NCAM-associated polysialic acid is variably expressed in different cell types, and is only weakly expressed in fibroblasts, thus underlying the much lower uptake of the peptide in these cells [20]. Third, signal-peptide-based cellular import is not based on endosomal uptake, as inhibitors of the endosomal pathway do not block it [4]. It does not depend on membrane caveolae, as it takes place in human T lymphocytes, which lack these membrane structures [19••,22]. Fourth, cellular import of nonfunctional signal kaposi peptide seems to be ATP-independent: its presence was observed in NIH3T3 cells depleted of their metabolic ATP pool [4]. Fifth, the cellular import of synthetic peptides is temperature-dependent and also requires the intact architecture of the plasma membrane, as treatment with glutaraldehyde, which disrupts the membrane, prevents peptide import [4]. Precisely how membrane fluidity and the lateral mobility of membrane proteins influence cellular import of synthetic peptides remains to be determined.

Applications of noninvasive delivery of peptides for the blockade of nuclear import of karyophilic proteins

The nuclear import of many karyophilic proteins, including DNA-binding transcription factors and viral proteins, depends on a short peptide sequence known as the nuclear localization signal (NLS). Typically, NLS motifs represent a cluster of basic amino acids (lysine and arginine residues) flanked by acidic and/or proline residues [23,24]. Karyophilic proteins containing an unmasked NLS are recognized in the cytoplasm by members of a growing family of NLS-binding proteins known as importins or karyopherins [25]. A newly formed karyophilic protein–importin/karyopherin complex is then bound to the cytoplasmic face of the nuclear pore before the NLS-bearing protein is shuttled into the nucleus. Thus, the NLS motif serves as a 'recognition signal' for nuclear import. In colaboration with Lin *et al.* [4], we have demonstrated effective blockade of nuclear import by noninvasive delivery of the cell-permeable SN50 peptide (which carries the NLS motif of the transcription factor NF-κB p50) to murine endothelial and human monocytic cell lines stimulated with proinflammatory agonists, lipopolysaccharide and tumor necrosis factor α. As a result, transcription factor NF-κB, which controls expression of numerous genes involved in the inflammatory and immune responses and in oxidant stress, was not imported to the nucleus. This noninvasive method of introducing NLS-bearing cell-permeable peptides in order to block nuclear import of karyophilic proteins was expanded to other transcription factors involved in regulation of the immune response in T cells [4].

The SN50 peptide blocked inducible nuclear import of NF-κB, AP-1 (activator protein 1), STAT1 and NF-AT (nuclear factor of activated T cells), which are transcription factors known to be imported into the nucleus and to be essential for gene transcription in activated T lymphocytes [19••]. Inhibition of nuclear import of these transcription factors resulted in attenuation of interleukin-2 (IL-2) gene expression in the human T cell Jurkat line. In addition, expression of *MAD3*, another gene encoding the NF-κB inhibitor IκBα, was also decreased [19••]. The functional sequence present in the SN50 peptide binds to importin-α (also known as Rch1 or karyopherin α) [19••]. These results indicate that the inducible nuclear import of four distinct families of transcription factors can be regulated by SN50 peptide delivered noninvasively to the cytoplasma of Jurkat T cells. In other studies [26], SN50 peptide was utilized to study contributions of NF-κB to excitoxin-induced apoptosis in rat brain striatum. (Excitocin is a substance that causes stimulation of neurons.) The SN50 peptide inhibited internucleosomal DNA fragmentation and striatal cell death, normal cellular responses to an apoptotic signal; however, this salutory effect may not be solely attributed to inhibition of nuclear import of NF-κB in view of the recent results in T lymphocytes demonstrate effective inhibition of other transcription factors [19••]. The SN50 peptide was also reported to inhibit nuclear import of NF-κB induced by interaction of the collagen lattice in human fibroblast cells with its receptor (which contains integrin α_2) [27]. Moreover, in human endothelial cells, SN50 peptide inhibited expression of IL-8 and

monocyte chemoattractant protein-1 induced by the membrane attack complex of complement [28]. Furthermore, a cell-permeable peptide containing different NLSs derived from the transcription factor upstream stimulating factor 2 inhibited its nuclear import in activated mast cells [29]. These examples illustrate the increasing range of applications of noninvasive delivery of peptides to block effectively nuclear import of karyophilic proteins.

Application of noninvasive intracellular delivery for structure/function analysis of integrin signaling

Cell–cell interactions and the attachment of cells to the extracellular matrix are mediated by integrins, major two-way signaling receptors that underlie developmental programming, immune responses, tumor metastasis and progression of atherosclerosis and thrombosis. While knowledge of the extracellular 'business end' of integrins, responsible for their binding to the extracellular matrix ligands and counter-receptors, is relatively well advanced, the intracellular 'business end', engaged in multiple regulatory interactions with signal tranducers and cytoskeletal components, is less well understood [30]. The cell-permeable peptide importation method was first applied to the structure/function analysis of the cytoplasmic tails of integrin $\alpha_{IIb}\beta_3$ and integrin $\alpha_v\beta_3$, which are endogenously expressed in the human erythroleukemia (HEL) cell line and human endothelial (ECV) cell line, respectively [5]. These structure/function studies, using cell-permeable peptide import, led to the identification of a novel cell adhesion regulatory domain (CARD) encompassing residues 747–763 in the cytoplasmic tail of integrin β_3, which modulates the interaction of integrins $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ with immobilized fibrinogen and the extracellular matrix.

Significantly, cell-permeable peptides that possess CARDs inhibited cell adhesion from 'within'. Moreover, inhibition of cell adhesion was integrin β_3-specific: a cell-permeable peptide homolog encompassing residues 788–803 of the cytoplasmic tail of integrin β_1 was inactive (i.e. did not inhibit cell adhesion) in an adhesion assay mediated by integrin β_3 (in HEL and ECV cells). Conversely, a cell-permeable peptide representing a homologous segment of the integrin β_1 cytoplasmic tail (comprising residues 788–803) inhibited adhesion of human fibroblasts mediated by integrin β_1 heterodimers, whereas a cell-permeable integrin β_3 peptide (comprising residues 747–762) did not produce this effect. Thus, two homologous CARDs, one present in the integrin β_3 (residues 747–763) and the other in the integrin β_1 cytoplasmic tail (residues 788–803), were identified using cell-permeable peptides [5]. Recently, cell-permeable peptides prepared via nonpeptide thiazolidine linkage were equally active to those described above in terms of their intracellular delivery and functional effect on integrin-mediated adhesion of HEL cells [6••].

Other applications of noninvasive delivery of peptides and proteins using the signal sequence hydrophobic core region

Rojas *et al.* [31] have applied the cell-permeable peptide importation method to control epidermal growth factor (EGF)-stimulated Ras activation in NIH3T3 cells overexpressing human EGF receptors. A cell-permeable peptide carrying one phosphotyrosine-containing segment of the EGF receptor representing its autophosphorylation site (Tyr1068) inhibited Ras activation and activation of its downstream effector mitogen-activated protein kinase (MAPK). Most recently, Rojas *et al.* [7••] extended these studies by preparing a recombinant 41 kDa fusion protein containing GST, the SH2 domains of the adaptor protein Grb2 and the modified h region of the signal peptide. This 12-amino acid motif called the membrane-translocating sequence differs from the original MPS [4] in that four residues are deleted. Membrane-permeable fusion proteins, upon noninvasive entry into NIH3T3 cells, formed a complex with the phosphorylated (i.e. activated) EGF receptor and inhibited EGF-induced signaling [7••].

Conclusions

Noninvasive cellular import of functional peptides and protein segments can be effectively used to probe signal transduction pathways involving intracellular domains of receptors such as integrins, receptor kinases (such as the EGF receptor), nonreceptor kinases (such as MAPKs), intracellular proteases (such as caspases) and transcription factors (such as NFκB). In some situations, the known functional domains (e.g. the NLS) can be imported, for example in the SN50 peptide, to block signaling to the nucleus by multiple transcription factors. In others, the cellular import of peptides provides the opportunity to conduct a detailed structure/function analysis of cytoplasmic segments of integrins and other receptors. Peptide mimetics of functionally relevant motifs can be used to block intracellular protein–protein interactions. The utility of noninvasive delivery of cell-permeable peptides and proteins based on the h region of signal peptides has been firmly established and offers a vast array of applications in probing and blocking intracellular protein–protein and protein–DNA interactions. A wide range of cell types, the speed and ease of translocation across the plasma membrane, free movement to cytoplasmic target proteins, low immunogenicity, and easy detectability of cell-permeable peptides overcome inherent limitations of currently used invasive methods such as microinjection of individual cells or the use of membrane-permeabilizing reagents.

Undoubtedly, these characteristics will be enhanced as ongoing studies expand the utility of the noninvasive delivery of peptides using the facile method of site-specific ligation of two free peptide modules. This will accelerate preparation of cell-permeable peptide libraries, mimetics and nonpeptide bioactive molecules. Moreover, importation of recombinant proteins is fully feasible. The ongoing investigation of the fundamental mechanism of membrane translocation,

subcellular distribution and turnover, and potential cytotoxicity of imported molecules will shed light on the mechanism of all attempted noninvasive methods of delivery of bioactive molecules into living cells. Noninvasive cellular import of bioactive molecules for their selective *in vivo* delivery to different types of cells is currently being developed.

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Intracellular translocation of the decapeptide carboxyl terminal of $G_i3\alpha$ induces the dual phosphorylation of p42/p44 MAP kinases

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Abstract

The carboxyl terminal of heterotrimeric G protein alpha subunits binds both G protein-coupled receptors and mastoparan (MP), a tetradecapeptide allostere. Moreover, peptides corresponding to the carboxyl domains of $G_i3\alpha$ and G_t display intrinsic biological activities in cell-free systems. Thus, the purpose of this study was to develop a cell penetrant delivery system to further investigate the biological properties of a peptide mimetic of the $G_i3\alpha$ carboxyl terminal ($G_i3\alpha^{346-355}$; H-KNNLKECGLY-NH_2). Kinetic studies, using a CFDA-conjugated analogue of $G_i3\alpha^{346-355}$, confirmed the rapid and efficient intracellular translocation of TP10-$G_i3\alpha^{346-355}$ ($t_{0.5}$=3 min). Translocated $G_i3\alpha^{346-355}$, but not other bioactive cargoes derived from PKC and the CB_1 cannabinoid receptor, promoted the dual phosphorylation of p42/p44 MAPK without adverse changes in cellular viability. The relative specificity of this novel biological activity was further confirmed by the observation that translocated $G_i3\alpha^{346-355}$ did not influence the exocytosis of β-hexoseaminidase from RBL-2H3, a secretory event stimulated by other cell penetrant peptide cargoes and MP. We conclude that TP10-$G_i3\alpha^{346-355}$ is a valuable, non-toxic research tool with which to study and modulate signal transduction pathways mediated by heterotrimeric G proteins and MAPK.

Keywords: Mastoparan; Secretion; p42/p44 MAP kinase; G protein; Cell penetrating peptide; Signalling

1. Introduction

A variety of cell-penetrating peptides (CPP) have been utilised as vectors for the intracellular delivery of bioactive cargoes to eukaryotic cells (reviewed in [1]). Moreover, the delivery of peptide cargoes is a valid methodology with which to study and specifically modulate discrete protein: protein interactions [2,3]. Clearly, the activity of any translocated peptide cargo will depend upon both its free intracellular concentration and its binding affinity for an accessible protein target. Thus, an ideal CPP should possess high translocation efficiency at concentrations that have little or no influence on the biology or viability of eukaryotic cells [1–4]. Previous studies [2] have confirmed that transportan-10 (TP10), a chimeric CPP that is an analogue of MP extended at the amino terminal with a sequence derived from galanin [5], is able to deliver bioactive peptide cargoes at concentrations that promote exocytosis from mast cells and activate signal-regulated phospholipase D. One mechanistic advantage of the TP10 system is that, following translocation, peptide cargoes are liberated by the intracellular reduction of cystine [2,5,6]. Hence, in this study, we further utilised TP10 as a CPP to affect the intracellular delivery of peptide cargoes.

The decapeptide sequence of the carboxyl terminal of $G_i3\alpha$, a component of inhibitory heterotrimeric G proteins, is conserved within human and rodent genomes [7]. A corresponding peptide has been used to confirm a role of

Abbreviations: CFDA, 5(-6)-carboxyfluorescein diacetate; $G_i3\alpha$, type 3 isoform of the α-subunit of the inhibitory guanine nucleotide binding protein; MP, mastoparan; TP10, transportan-10

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plasma membrane-bound $G_i3\alpha$ in the exocytotic response of melanotrophs [8]. Significantly, the $G_i3\alpha$ peptide, but not a corresponding sequence from $G_i1/2\alpha$, neutralised MP-induced changes in membrane capacitance when dialysed into rat pituitary melanotrophs using a patch clamp pipette [8]. Related peptides, introduced to permeabilized cell systems, have provided further evidence for a specific role of $G_i3\alpha$ in mediating MP-induced secretory events in both chromaffin cells [9] and peritoneal mast cells [10]. Collectively, these studies indicate that relatively short peptides can mimic the activities of heterotrimeric G proteins. Thus, the major aim of this study was to develop a biologically inert, non-toxic CPP-mediated delivery system that would enable the intracellular delivery and study of a peptide corresponding to the carboxyl terminal of $G_i3\alpha$ (H-KNNLKECGLY-NH_2; $G_i3\alpha^{346-355}$). The choice of this sequence was determined by our two recent investigations. Firstly, MP and a variety of analogues activate MAP kinase signalling and reduce the viability of U373MG astrocytoma, a cell line that constitutively expresses $G_i3\alpha$ [11]. This cellular system was used to determine whether translocated $G_i3\alpha^{346-355}$ could also modulate MAP kinase phosphorylation and/or cellular viability. As MP is believed to interact with the carboxyl-terminal of G protein α subunits [12], studies reported here also addressed the question of whether TP10-$G_i3\alpha^{346-355}$ could abrogate the biological activities of MP. Secondly, TP10-translocated peptides derived from PKC and the CB_1 cannabinoid receptor [2], and MP analogues [13], stimulate β-hexoseaminidase secretion from RBL-2H3 mast cells. Moreover, regulated secretion from mast cells is reported to be $G_i3\alpha$ dependent [10]. Hence, investigations determined if translocated $G_i3\alpha^{346-355}$ could also modulate mast cell secretion. To confirm effective cellular delivery, the translocation kinetics of a fluoresceindiacetate-conjugated analogue of $G_i3\alpha^{346-355}$ was determined using spectrofluorimetry.

Results presented herein indicate that the bipartite peptide TP10-$G_i3\alpha^{346-355}$ is a particularly useful tool to study the molecular mechanisms by which GPCRs activate p42/p44 MAP kinases, a cell-type specific phenomenon regulated by a complex of inter-connected signalling pathways [14].

2. Experimental procedures

2.1. Materials

N-α-*tert*-Butyloxycarbonyl-Cys(3-Nitro-2-pyridinesulfanyl)-OH (Boc-Cys(Npys)-OH) was from Bachem (St. Helens, UK). 5(-6)-Carboxyfluorescein diacetate succinimdyl ester (CFDA) was purchased from Molecular Probes (Leiden, Netherlands). All other materials for solid phase peptide synthesis were from Novabiochem (Beeston, UK). Cell culture medium was obtained from PAA Laboratories Ltd (Linz, Austria).

2.2. Peptide synthesis, purification and analysis

MP (H-INLKALAALAKKIL-NH_2), [Lys^{7Mtt}]TP10, $G_i3\alpha^{346-355}$ and other peptides (Fig. 1) were manually synthesized (0.1–0.2 mmol scale) on a Rink amide methylbenzhydrylamine resin employing an *N*-α-Fmoc protection strategy with HBTU/HOBt activation [2]. The selective removal of the 4-Methyltrityl group (Mtt) of [Lys^{7Mtt}]TP10 with TFA (3% v/v in DCM, 2 × 10 min), acylation with Boc-Cys(Npys) (2 equivalents) and cleavage with TFA/H_2O/triisopropylsilane (95%/2.5%/2.5%) yielded

A [$Lys^7N^{\varepsilon Cys(Npys)}$]TP10

H-Ala-Gly-Tyr-Leu-Leu-Gly-Lys-Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2

Cys(Npys)

B TP10-$G_i3\alpha^{346-355}$

H-Ala-Gly-Tyr-Leu-Leu-Gly-Lys-Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2

Cys

H-Lys-Asn-Asn-Leu-Lys-Gly-Cys-Gly-Leu-Tyr-NH_2

Other cargoes

$Cys^0PKC^{238-249}$ H-**Cys**-Arg-Arg-Leu-Ser-Val-Glu-Ile-Trp-Asp-Trp-Asp-Leu-NH_2

$CB_1^{401-417}$ H-Arg-Ser-Lys-Asp-Leu-Arg-His-Ala-Phe-Arg-Ser-Met-Phe-Pro-Ser-**Cys**-Glu-NH_2

Fig. 1. Sequences of [$Lys^{7N\varepsilon Cys(Npys)}$]TP10 and TP10-$G_i3\alpha^{346-355}$. The Npys group of [$Lys^{7N\varepsilon Cys(Npys)}$]TP10 reacts rapidly with free thiols to direct disulfide bond formation with Cys-containing peptides in high yields. The disulfide bond of TP10-$G_i3\alpha^{346-355}$ (B) is reduced after cellular translocation to liberate the free cargo. The sequences of other cargoes used in this study (see also Figs. 2 and 3) are derived from an autoregulatory domain of PKC ($Cys^0PKC^{238-249}$) and an intracellular domain of the CB_1 cannabinoid receptor ($CB_1^{401-417}$). Cys residues in bold indicate the position of attachment to TP10 in these sequences.

the fully deprotected [Lys$^{7N\varepsilon Cys(Npys)}$]TP10. The peptide cargo H-KNNLKECGLY-NH_2, a sequence that mimics the extreme decapeptide carboxyl terminal of $G_i3\alpha$, was synthesized with an amidated carboxyl terminal to confer increased resistance to proteolysis. Fluorescent labelling of this sequence to generate [CFDA]$G_i3\alpha^{346-355}$ was achieved by acylation of the lysine amino terminal with CFDA succinimide ester. The conjugation of [Lys$^{7N\varepsilon Cys(Npys)}$]TP10 to cargoes $G_i3\alpha^{346-355}$ and [CFDA]$G_i3\alpha^{346-355}$ (Fig. 1) was achieved by dissolving [Lys$^{7N\varepsilon Cys(Npys)}$]TP10 and cargoes (2- to 3-fold molar ratio) in a minimum volume of DMF/DMSO/0.1 M $C_2H_3O_2Na$, pH 5.0 (3/1/1), and mixing overnight (Ursel Soomets, personal communication). All peptides were purified by semi-preparative scale, high-performance liquid chromatography and lyophilized [2]. The predicted masses of all peptides used in this study (average $M+H^+$) were confirmed to an accuracy within ±1 by matrix-assisted laser desorption ionisation time of flight mass spectrometry (Kratos *Kompact Probe* operated in a positive ion mode).

2.3. Cell culture

U373MG astrocytoma and RBL-2H3 basophilic leukaemia cells were maintained in DMEM containing L-glutamine (0.1 mg ml^{-1}) and supplemented with foetal bovine serum (10% v/v), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.4. Detection of p42/p44 MAP kinases by Western immunoblotting

U373MG cells were cultured as described above in 6-well plates. To reduce basal levels of p42/p44 phosphorylation, cells were washed and maintained in a medium without serum for 24 h prior to the addition of peptides [11]. Cells were exposed to peptides or serum and reactions were terminated by the addition of an ice-cold lysis buffer (20 mM HEPES (pH 7.2), 50 mM NaF, 1 mM Na_3VO_4, 50 mM β-glycerophosphate, 1 mM EGTA, 1% (v/v) Triton X-100 and protease inhibitor cocktail tablets containing EDTA (Roche)) at 4 °C for 15 min. Wells were scraped and lysates centrifuged for 15 min at 13,000×*g* at 4 °C to remove cell debris. Supernatants were subjected to SDS/PAGE in 10% gels. Proteins were electro-transferred to PVDF membrane and blocked overnight at 4 °C in a blocking solution (20 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween-20, 1% (w/v) BSA). To detect dually phosphorylated p42/p44 MAP kinases, membranes were probed with primary rabbit anti-active MAPK antibody (Promega). Total p42/p44 MAP kinases were identified in the same cell lysates using primary rabbit anti-ERK1/2 antibody (Promega). Protein–antibody conjugates were probed with secondary antibody (donkey anti-rabbit, conjugated to horseradish peroxidase, Promega) and visualised by enhanced chemiluminescence. The quantification of signal intensities (mean O.D.) utilised a Scion Image Beta software (Scion Image Corp., Frederick, Maryland).

2.5. Analysis of peptide-induced β-hexoseaminidase secretion

Assays were performed as previously described [2,11,13]. To facilitate the maximal delivery of peptide cargoes, confluent cells in 24-well plates were treated with TP10 chimeras for a period of 60 min at a maximum concentration of 3 μM [2]. Following the exposure of cells to peptides, samples of the medium (5 μl) were transferred into 96-well plates and incubated with ρ-nitrophenyl *N*-acetyl-β-D-glucosamide (20 μl of 1 mM in 0.1 M sodium citrate buffer, pH 4.5) for 1 h at 37 °C. $Na_2CO_3/NaHCO_3$ buffer (200 μl of 0.1 M, pH 10.5) was then added and β-hexosaminidase activity determined by colourimetric analysis at 405 nm.

2.6. Kinetic analysis of TP10-[CFDA]$G_i3\alpha^{346-355}$ translocation

Confluent RBL-2H3 cell cultures in 75 cm^2 flasks were washed with and scraped into 5 ml balanced salt solution (BSS). Cells were collected by centrifugation, suspended in 1 ml of BSS, and transferred to a stirred, temperature-regulated quartz cuvette in a Hitachi F-2500 fluorescence spectrophotometer at 37 °C. Following the intracellular delivery of [CFDA]$G_i3\alpha^{346-355}$, as a TP10 conjugate, esterase activity cleaved CFDA to generate the fluorescein chromophore that was quantitatively detected using excitation/emission wavelengths of 495 nm and 525 nm respectively. The specific fluorescent signal generated by translocated TP10-[CFDA]$G_i3\alpha^{346-355}$ was calculated at six time points by subtracting background fluorescence from the composite signal.

2.7. Cell viability assays

The viability of the U373MG cells was measured by the 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) conversion assay [11,15]. Cells were cultured as above in 96-well plates and washed and maintained in a medium without serum for 24 h prior to assay. Cells were treated with peptides for 4 h at 37 °C and further incubated with MTT (0.5 mg ml^{-1}) for 3 h at 37 °C. The medium was aspirated and the insoluble formazan product was solubilized with DMSO. MTT conversion was determined by colourimetric analysis at 540 nm.

2.8. Statistical analysis

Where indicated, the Mann–Whitney test was used to determine the significance of measured changes in cellular viability.

3. Results

3.1. Translocated $G_i3\alpha^{346-355}$ activates a signal transduction cascade that terminates in the dual phosphorylation of p42/p44 MAP kinases

We have recently reported [11] that MP and a range of structural homologues induce the dual phosphorylation of p42/p44 MAP kinases in U373MG cells. As secretory events complicate the temporal analysis of MAP kinase activation in RBL-2H3 [11], the studies presented here also used the U373MG astrocytoma line. We anticipated (see Introduction) that the intracellular delivery of $G_i3\alpha^{346-355}$ would influence MP-induced MAP kinase activation in U373MG. 30 min of exposure of U373MG cells to MP (3–30 μM; Fig. 2A) produced a marked increase in dually phosphorylated p42/p44 MAP kinases. Prior incubation of cells with 3 μM TP10-$G_i3\alpha^{346-355}$ (indicated as TP10-$G_i3\alpha$ in Fig. 2A) for 30 min did not reduce signal intensity in MP-treated cells.

TP10-$G_i3\alpha^{346-355}$, but not unconjugated $G_i3\alpha^{346-355}$, also activated a signal transduction cascade leading to the dual phosphorylation of p42/p44 MAP kinases. The treatment of cells with TP10-$G_i3\alpha^{346-355}$ (indicated as TP10-$G_i3\alpha$ in Fig. 2B), for 60 min at a concentration of 3 μM, increased the phosphorylation of p42/p44 MAP kinases to levels comparable to those observed after treatment of cells with MP (Fig. 2A) or serum (FBS; Fig. 2C). The cell penetrant vector [$Lys^{7N\varepsilon Cys}$]TP10 produced a minor increase in signal intensity, whilst $G_i3\alpha^{346-355}$ added alone was inactive. A comparison of signal intensities indicated that both MP and TP10-$G_i3\alpha^{346-355}$ maximally activated the phosphorylation of p42/p44 to levels comparable to those induced by serum (Fig. 2C). Quantitative analyses of these data (mean pixel intensity) confirmed that unconjugated $G_i3\alpha^{346-355}$ produced no change in signal intensity whilst both [$Lys^{7N\varepsilon Cys}$]TP10 and TP10-$G_i3\alpha^{346-355}$ increased signal intensities by 1.7-fold and 5.0-fold over basal levels.

The specificity of the $G_i3\alpha$-derived peptide cargo was confirmed (Fig. 2C) by comparison with two other peptide cargoes, derived from PKC and the CB_1 cannabinoid receptor, respectively, that activate β-hexoseaminidase secretion (Fig. 1; [2]). Incubation of cells with these unconjugated peptides had no influence on the phosphorylation state of MAP kinases. Moreover, the intracellular delivery of these sequences, as TP10 chimera, did not stimulate the dual phosphorylation of p42/p44 MAP kinases to levels above those observed with vector alone.

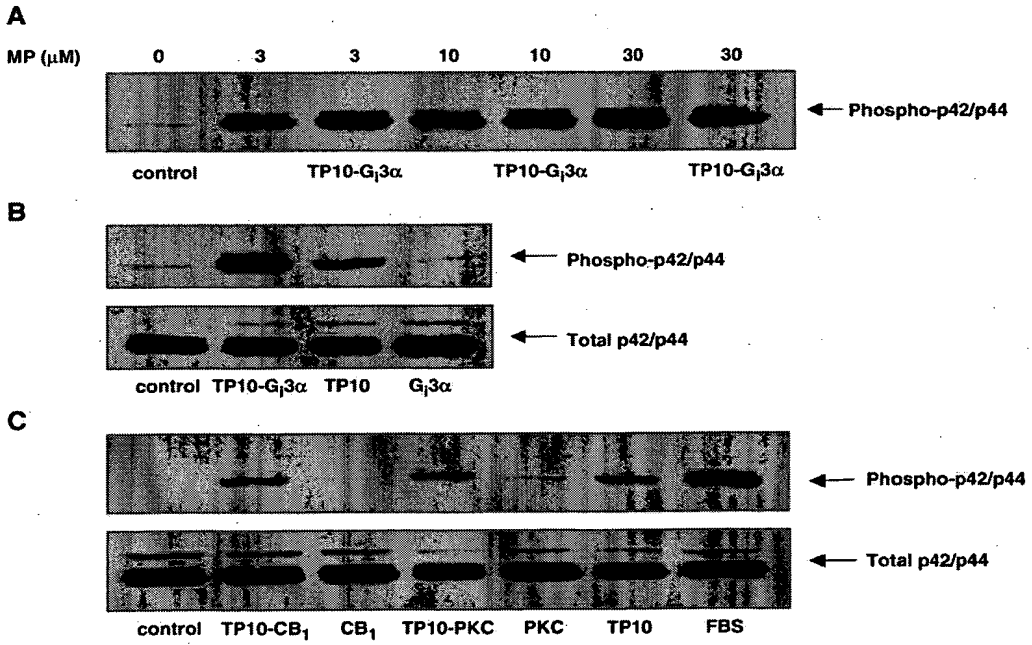

Fig. 2. The phosphorylation state of p42/p44 MAP kinase is regulated by bioactive peptides. The concentrations of peptides used and exposure times were as previously reported [2,11] and optimized for p42/p44 detection in U373MG astrocytoma. We have confirmed, in numerous investigations, that the treatment of U373MG with MP has no influence on the total p42/p44 MAP kinase content of cells ([11]; Jones, PhD thesis, University of Wolverhampton, 2005). (A) Cells were treated with MP for 30 min at range of concentrations from 3 to 30 μM. Where indicated as TP10-$G_i3\alpha$, cells were exposed to 3 μM TP10-$G_i3\alpha^{346-355}$ for 30 min prior to the addition of MP for an additional period of 30 min. (B) Treatment of cells with TP10-$G_i3\alpha^{346-355}$ (indicated as TP10-$G_i3\alpha$), vector [$Lys^{7N\varepsilon Cys}$]TP10 and unconjugated cargo $G_i3\alpha^{346-355}$. All peptides were added to cells at a concentration of 3 μM for 60 min. Bottom panel shows total levels of p42/p44 MAP kinases in the same cell lysates. (C) Cells were incubated with the following peptides at 3 μM for 60 min: TP10-$CB_1^{401-417}$ (TP10-CB_1); $CB_1^{401-417}$ (CB_1); TP10-$Cys^0PKC^{238-249}$ (TP10-PKC); $Cys^0PKC^{238-249}$ (PKC); and [$Lys^{7N\varepsilon Cys}$]TP10 (TP10). This figure also illustrates increased p42/p44 phosphorylation in response to the addition of 10% v/v serum (FBS) for 60 min. Bottom panel shows the total levels of p42/p44 MAP kinases in the same cell lysates.

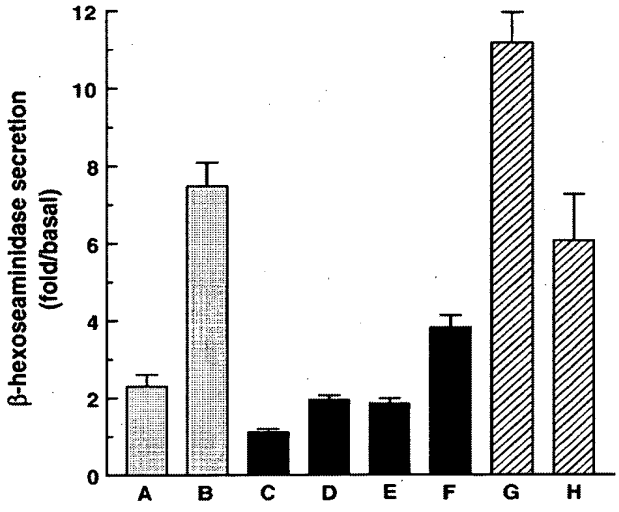

Fig. 3. Peptide-induced secretion of β-hexoseaminidase from RBL-2H3 mast cells. This figure compares the fold/basal secretory activities of peptides and peptide combinations (A–H). The times of exposure to various peptides are optimal and based on our previous studies with MP analogues [11,13] and other cell penetrant constructs using TP10 as a delivery vector [2]. All data are means±S.E.M. from at least three experiments each performed in triplicate. Legend: (A) MP (10 μM, 15 min); (B) MP (30 μM, 15 min); (C) $G_i3\alpha^{346-355}$ (3 μM, 60 min); (D) [$Lys^{7N\varepsilon Cys}$]TP10 (3 μM, 60 min); (E) TP10-$G_i3\alpha^{346-355}$ (3 μM, 60 min); (F) TP10-$G_i3\alpha^{346-355}$ (3 μM, 45 min)+MP (10 μM, 15 min); (G) TP10-$Cys^0PKC^{238-249}$ (3 μM, 60 min); and (H) TP10-$CB_1^{401-417}$ (3 μM, 60 min).

3.2. TP10-$G_i3\alpha^{346-355}$ does not reduce cellular viability

The exposure of U373MG cells to the MAP kinase activator MP concentration dependently reduces cellular viability [11]. Concentrations of MP that activate MAP kinase (3–30 μM) significantly reduce cellular viability in a 4-h time period [11]. As signalling by GPCRs, MAP kinase activation and cell fate are intrinsically linked at many levels (reviewed in [16]), we also determined whether exposure of U373MG to TP10-$G_i3\alpha^{346-355}$ and its components, at a concentration used for effective cellular penetration, might also reduce cellular viability. Viability data (percentage viability, mean±S.E., $n=6$) were determined following exposure of cells to peptides for 4 h, and values were normalised to those of control cells: control cells (100); MP (30 μM), 47.4±4.4; TP10-$G_i3\alpha^{346-355}$ (3 μM), 110.0±4.0; [$Lys^{7N\varepsilon Cys}$]TP10 (3 μM), 108.6±5.9; $G_i3\alpha^{346-355}$ (3 μM), 85.4±9.4. Only MP produced a significant change in cellular viability ($P=0.0022$, Mann–Whitney test).

3.3. Intracellular delivery of $G_i3\alpha^{346-355}$ does not modulate β-hexoseaminidase secretion from RBL-2H3

We have demonstrated that the secretion of β-hexoseaminidase from RBL-2H3 in response to MP analogues is concentration-dependent, but this exocytotic process is insensitive to the exogenous application of many other polybasic peptides [13]. This secretory pathway in RBL-2H3 is activated by TP10 chimeric constructs that translocate peptide sequences (Fig. 1) derived from an autoregulatory domain of PKC and the third intracellular loop of the CB_1 cannabinoid receptor [2]. Therefore, and with reference to previous observations using peptides derived from the $G_i3\alpha$ carboxyl-terminal [8–10], we anticipated that TP10-$G_i3\alpha^{346-355}$ would reduce the magnitude of MP-induced secretion of β-hexoseaminidase (Fig. 3, bars A and B). When added alone, $G_i3\alpha^{346-355}$ had no effect on β-hexoseaminidase secretion (Fig. 3, bar C; $P>0.5$, Student's t-test). All other secretory responses (Fig. 3, bars D–H) were significant ($P<0.05$) compared with basal levels of secretion that represented <5% of total cellular β-hexoseaminidase in a 1-h incubation. Both the cell penetrant vector [$Lys^{7N\varepsilon Cys}$]TP10 (Fig. 3, bar D) and TP10-$G_i3\alpha^{346-355}$ (Fig. 3, bar E) were weak secretagogues. The addition of TP10-$G_i3\alpha^{346-355}$ followed by MP produces an additive effect on the magnitude of β-hexoseaminidase secretion (Fig. 3, bar F). This figure also includes comparative data showing the magnitude of secretory responses to TP10-$Cys^0PKC^{238-249}$ (Fig. 3, bar G) and TP10-$CB_1^{401-417}$ (Fig. 3, bar H), as previously reported [2]. The secretory efficacies of TP10-$G_i3\alpha^{346-355}$ and the unconjugated vector [$Lys^{7N\varepsilon Cys}$]TP10 were similar and clearly a consequence of the MP-containing sequence of the carboxyl terminal of TP10 [10]. These observations indicate that $G_i3\alpha^{346-355}$ does not directly influence the biochemical pathway leading to the exocytosis of β-hexoseaminidase or abrogate the secretory activity of MP.

3.4. Translocation kinetics of TP10-$G_i3\alpha^{346-355}$

The confirmation that TP10-[CFDA]$G_i3\alpha^{346-355}$ effectively penetrated RBL-2H3 cells was obtained using real-time fluorescence spectroscopy. As indicated in Fig. 4, the addition to cells of the non-fluorescent and non-permeable peptide [CFDA]$G_i3\alpha^{346-355}$ (3 μM) produced a gradual,

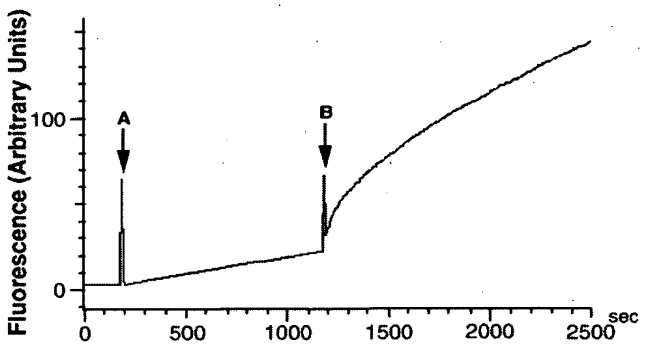

Fig. 4. Fluorimetric analysis of TP10-[CFDA]$G_i3\alpha^{346-355}$ translocation. This figure, representative of two others, indicates the metabolic generation of the fluorescein chromophore following the internalisation of TP10-[CFDA]$G_i3\alpha^{346-355}$. Arrow A indicates the addition of unconjugated [CFDA]$G_i3\alpha^{346-355}$ (3 μM) to RBL-2H3 cells at 37 °C. Arrow B marks the further addition of TP10-[CFDA]$G_i3\alpha^{346-355}$ (3 μM). Ordinate: fluorescence intensity (arbitrary units Ex/Em 495/525 nm); Abscissa: time (s).

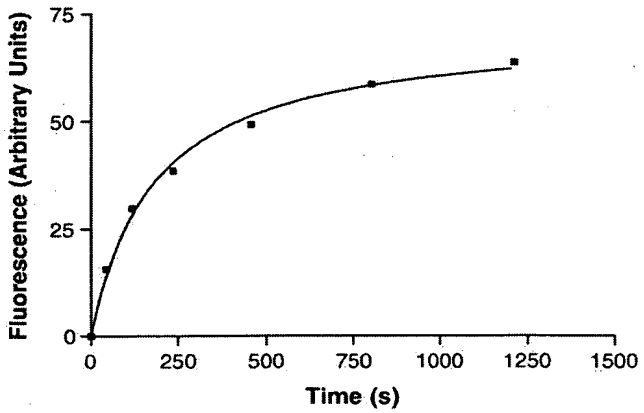

Fig. 5. Kinetic analysis of TP10-mediated translocation. In this figure, time 0 equates to the addition of TP10-[CFDA]$G_i3\alpha^{346-355}$ as indicated by arrow B in Fig. 4. Ordinate: fluorescence intensity (arbitrary units Ex/Em 495/525 nm); Abscissa: time (s).

linear increase in fluorescence over a time period of some 100 s that probably reflects the activity of extracellular esterases. This change in signal intensity was quantified at Δ0.019 units/s. The further addition of TP10-$G_i3\alpha^{346-355}$ (3 μM) produced a rapid and marked increase in fluorescence signal indicative of effective cellular penetration. To further analyse these data, the specific fluorescent signal (F) generated by internalised [CFDA]$G_i3\alpha^{346-355}$ was calculated at six time points by subtracting background fluorescence from the composite signal. Background fluorescence was calculated at Δ0.038 units/s from the time of addition of TP10 [CFDA]$G_i3\alpha^{346-355}$ to cells as indicated by arrow B on Fig. 4. These data were analysed using Fig. P software (Biosoft) and indicate that internalisation occurred with first order kinetics ($F=F_{max}\times t/t_{0.5}+t$) with a half life ($t_{0.5}$) of 180.8 s and a maximum signal intensity (F_{max}) of 71.5 units (Fig. 5).

4. Discussion

The intention of this study was to develop an inert, non-toxic, cell penetrant system to affect the intracellular delivery of a peptide mimic of the extreme carboxyl terminal of $G_i3\alpha$. Thus, the bipartite TP10-$G_i3\alpha^{346-355}$ chimera was designed to deliver a peptide sequence similar to that previously reported to ablate the secretory action of MP [13,15]. It is currently uncertain whether the carboxyl termini of G protein α subunits are subject to post-translational modifications. Thus, the $G_i3\alpha^{346-355}$ peptide cargo was synthesized with an amidated carboxyl terminal to reduce intracellular proteolysis. The selection of TP10 as a cell penetrant vector was dictated by previous studies that indicate TP10 to be relatively inert [5] and capable of delivering peptide cargoes to RBL-2H3, a cell line that is usually responsive to MP-containing sequences [2,11,13]. The amino terminal extension of MP in TP10 (galanin(7-12)-Lys) both reduces the ability of the MP-containing carboxyl segment to modify GTPase activity, a feature characteristic of other MP sequences, and enhances efficient translocation [5]. Three different biological assay systems were employed to rigorously assess the utility of our novel TP10-$G_i3\alpha^{346-355}$ construct. Collectively, these assays assessed MAP kinase activation, cellular viability and regulated secretion to also enable comparison with MP, a well-documented direct activator of G proteins [17,18].

The intracellular delivery of $G_i3\alpha^{346-355}$, as a TP10 chimeric construct, promoted a kinase cascade that terminated with the dual phosphorylation of p42/p44 MAP kinases. These data provide evidence that the decapeptide carboxyl-terminal of $G_i3\alpha$ contributes to both the binding and activation of intracellular target proteins. Support for this contention is provided by previous studies [19,20] indicating that a peptide derived from the carboxyl domain of transducin ($G_t\alpha^{293-314}$) binds and activates cGMP-phosphodiesterase, a novel intracellular effector of rhodopsin–transducin signalling. Moreover, an undecapeptide mimetic of the extreme carboxyl terminal of tranducin ($G_t\alpha^{340-350}$), and sequence variants, also binds light-activated rhodopsin with high affinity [21]. Thus, in addition to an interaction with effector proteins, the relatively mobile carboxyl-terminal of G protein α subunits also regulates GDP dissociation [23] and contributes to the binding of both GPCRs [21–24] and the receptor-mimetic peptide MP [12]. Our findings provide direct evidence that a component of the MAP kinase signalling pathway is a molecular target for activated $G_i3\alpha$ in U373MG cells. This interpretation is in accord with reports that both the α and βγ subunits of heterotrimeric G proteins can activate signalling events that lead to the dual phosphorylation of p42/p44 MAP kinases [14]. MP and sequence variants also activate p42/p44 MAP kinases in U373MG whilst Mas17, a structural analogue with much reduced efficacy, is inactive. Thus, translocated $G_i3\alpha^{346-355}$ mimics one action of MP, suggesting that this decapeptide binds and activates a common intracellular molecular target of GTP-bound $G_i3\alpha$. Moreover, $G_i3\alpha$ is constitutively expressed in U373MG and is, therefore, a potential target for MP that is reported to bind both the carboxyl [12] and amino [25] termini of G protein α subunits. However, as further discussed below, $G_i3\alpha^{346-355}$ does mimic other biological actions of MP that may be a consequence of the ability of MP to modulate the activity of other intracellular targets. These results also demonstrate that unconjugated $G_i3\alpha^{346-355}$ is unable to cross an intact plasmalemma but is biologically active following intracellular translocation as a TP10 chimera. These investigations further confirm the utility of the TP10 system for the efficient intracellular delivery of peptide cargoes that are liberated by intracellular reduction of the disulfide linkage following cellular penetration [2,5,6].

A general requirement of CPP technology is that the delivery vector of choice should be non-toxic. MAP kinase

activation can influence cell fate to promote differentiation or apoptosis, disparate outcomes that are seemingly dependent upon signal strength and duration [16,26]. Moreover, MP, a component of TP10, also reduces U373MG cell viability in a concentration-dependent manner [2]. Thus, it was important to determine whether TP10-$G_i3\alpha^{346-355}$ influenced cellular viability. Data presented here indicate that neither TP10-$G_i3\alpha^{346-355}$ nor unconjugated $G_i3\alpha^{346-355}$ reduced the viability of U373MG cells at a concentration (3 μM) that provides effective cellular delivery in the case of TP10–peptide conjugates. Furthermore, cellular viability was maintained for a minimum period of 4-h exposure to peptides, a time course much longer than that required to observe the biological effects of TP10-translocated peptide cargoes [2]. Hence, these data provide further evidence that the cytotoxic action of MP results from the interaction with other protein targets in addition to $G_i3\alpha$ [11], and this activity is not mimicked by translocated $G_i3\alpha^{346-355}$.

Significantly, $G_i3\alpha^{346-355}$ was without effect on β-hexoseaminidase exocytosis and did not influence the secretory activity of MP (see Fig. 3). Similar negative results were obtained when $G_i3\alpha^{346-355}$ was exogenously applied to cells or delivered to the intracellular environment as a TP10 chimera. These findings are contrary to previous reports [8,10] that have used similar peptides to block MP-induced secretory events; and we have recently demonstrated that other TP10–peptide chimeras, used to deliver peptides derived from PKC and the CB_1 cannabinoid receptor, are potent activators of β-hexoseaminidase secretion from RBL-2H3 [2]. One relatively simple explanation of these data could be that $G_i3\alpha^{346-355}$ does not prevent MP binding to the $G_i3\alpha$ protein. Alternatively, the lack of activity of $G_i3\alpha^{346-355}$ in RBL-2H3 could indicate that the G_i3 protein α subunit (or at least its carboxyl terminal) does not contribute to the exocytosis of secretory lysosomes. Considering the reported specificity and selectivity of action of peptides derived from the extreme carboxyl-termini of G protein α subunits [8], this interpretation would suggest that that the secretion of β-hexoseaminidase from RBL-2H3 is not a $G_i3\alpha$-dependent phenomenon. Indeed, recent observations have suggested that $G_i3\alpha$ is not the elusive exocytotic G protein (G_E) responsible for regulated secretory events in mast cells [27].

The use of [CFDA]$G_i3\alpha^{346-355}$ as a fluorescent probe proved to be a very effective strategy to determine the translocation kinetics of TP10. This approach relies on the fact that CFDA is non-fluorescent when exogenously added to cells, but is rapidly metabolised by intracellular esterases to unmask the fluorescein chromophore that is readily detected using spectrophotometry [28]. Moreover, the metabolism of CFDA occurs within cytosol, organelles and other intracellular compartments and is therefore suitable for the labelling of both CPP and cargoes that are differentially distributed following the translocation of the plasma membrane [4,6,29–32]. The data presented here provide an estimate of the rate of internalisation of TP10 ($t_{0.5}$) of approximately 3 min that is equivalent to previous estimates of 8 min in Bowes melanoma [5] and certainly faster than the reported endocytotic internalisation of the Tat CPP [29]. Whether there is indeed a common mechanism of translocation for the different classes of CPP is a subject of much on-going debate [1,29–33].

In summary, our data indicate that a cell penetrant mimetic peptide cargo, corresponding to the decapeptide carboxyl-terminal of $G_i3\alpha$, specifically stimulates a signal transduction pathway leading to the activation p42/p44 MAP kinases. In this regard, translocated $G_i3\alpha^{346-355}$ behaves as a selective analogue of the G protein activator MP, a peptide that is widely employed as an allosteric modulator of G proteins. Such an approach provides a general strategy to both study and manipulate cell signalling pathways, transduced via G proteins and divergent kinase cascades, that represent potential drug targets.

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